



## Review

## Estradiol measurement in translational studies of breast cancer



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## ABSTRACT

Plasma estrogen measurement with use of radioimmunoassays has been instrumental in the development of aromatase inhibitors for endocrine therapy of postmenopausal breast cancer. However, due to low plasma estrogen concentrations in postmenopausal women, direct radioimmunoassays lack the sensitivity required. While certain laboratories have developed highly sensitive assays for research purposes revealing plasma estrogen suppression consistent with results from tracer studies, such assays are time and labor-consuming due to need for pre-analytical chromatographic purification, sample concentration and sometimes conversion of precursors to products. While novel chromatographic methods involving mass spectrometry analysis are likely to replace such radioimmunoassays in the future, so far a limited number of laboratories have developed suitable assays with a detection limit (around 1 pM) that is required for analyzing plasma estrogen levels in patients during treatment with potent aromatase inhibitors.

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## 1. Introduction

While the history of endocrine therapy in breast cancer started more than a century ago, the scientific rationale arose with the discovery of [1] and confirmation of the predictive value of [2] the estrogen receptor (today known as ER $\alpha$ ). In parallel, introduction of what was known at that time as an adrenotoxic antiepileptic, aminoglutethimide, in an attempt to achieve a “medical adrenalectomy” [3], indirectly led to introduction of aromatase inhibition as a successful endocrine therapy for postmenopausal women. While clinically efficient [4], subsequent studies by the Hershey group

revealed sustained androgen production, despite estrogen suppression [5], a question solved when tracer studies [6] revealed that aminoglutethimide directly inhibited peripheral aromatization of androstenedione into estrone (E<sub>1</sub>). The discovery that aminoglutethimide executed its anti-tumor efficacy through aromatase inhibition had profound influence on subsequent development of endocrine therapy for breast cancer. While effective, aminoglutethimide treatment was associated with substantial side effects [7], for which reason much efforts were placed on developing novel, less toxic agents (see Ref. [8]). Importantly, the successful implementation of aromatase inhibition as a major endocrine for breast cancer, from which more than a million breast cancer women around the world currently are benefitting, had not been possible without these careful translational studies applying sensitive radioimmunoassay's, in concert with tracer studies, to define the

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pharmacological mechanisms of action of aminoglutethimide and, thus, aromatase inhibition as a useful endocrine treatment option in breast cancer.

## 2. Tracer studies

While tracer studies and plasma estrogen assessment in concert confirmed aminoglutethimide to act as a potent aromatase inhibitor *in vivo* [9], plasma estrogen radioimmunoassay's lacked the sensitivity to detect estrogen suppression >90%, which was what to expect based on the tracer results. Thus, direct assessment of *in vivo* aromatase inhibition by tracer studies were considered to be the “gold standard”. With new aromatase inhibitor compounds in development, in collaboration with Professor Mitch Dowsett and his team, we initiated a program for *in vivo* assessment of aromatase inhibition. Using an HPLC method to separate estrogen metabolites [10], we developed an assay allowing assessment of *in vivo* aromatase inhibition with an average detection limit >99.1% [11–18]. The results are depicted in Table 1; in brief, while most compounds (the so-called 1st and 2nd generation compounds) caused *in vivo* aromatase inhibition <90%, the three 3rd generation compounds; the steroidal inhibitor exemestane, as well as the non-steroidal compounds anastrozole and letrozole, each caused on average >98% aromatase inhibition. Most importantly; these endocrine results were paralleled by clinical findings; while the 1st and 2nd generation compounds in general revealed clinical efficacy similar to tamoxifen [8], the three 3rd generation compounds revealed superiority, also with respect to clinical efficacy, and are today used as routine endocrine therapy for postmenopausal women in the adjuvant setting [19].

## 3. Plasma estrogen measurements in relation to treatment with aromatase inhibitors

The results from *in vivo* tracer studies, in concert with the results from large randomized studies, underline the imperative of maximal aromatase inhibition for optimal clinical efficacy. Thus, while randomized studies revealed superiority for third-generation aromatase inhibitors as compared to tamoxifen [19], in contrast first- and second-generation compounds were found of similar efficacy, but not superior, as compared to conventional therapy [8,20]. Tracer studies however are laborious and expensive to conduct and may be applied to small patient groups only. Thus, there is a need for simpler methods, like plasma estrogen measurement.

A key problem relates to low plasma estrogen levels in postmenopausal women, in particular when on aromatase inhibitor

therapy. Taking into account plasma levels of estradiol ( $E_2$ ),  $E_1$  and estrone sulfate ( $E_1S$ ) to be in the 15–20 pM, 70–80 pM and 4–500 pM ranges, respectively [21], the assays need sensitivity limits of a few pM to detect potential suppression >98%. Developing highly sensitive radioimmunoassays with a detection limit of about 1 pg/ml (3.7 pM), the Herhsey group revealed significant differences with respect to plasma estrogen suppression between the second-generation aromatase inhibitor CGS16949A [22] and letrozole [23]. Notably, as for both studies the difference in plasma estrogen suppression was corroborated by similar findings with respect to suppression of urinary estrogen secretion.

A problem related to use of  $^3H$ -labelled standards in radioimmunoassays relates to the limited specific activity of these standards (in the 50–160 mCi/mmol range). A higher specific activity may be achieved with use of  $^{125}I$ -labelled compounds (specific activity in the 2000 Ci/mmol range). The first sensitive  $^{125}I$ -based RIA for  $E_2$  measurement in patients treated with aromatase inhibitors was developed by Professor Mitch Dowsett at the Royal Marsden Hospital [24] and subsequently used to measure plasma  $E_2$  suppression with different aromatase inhibitors [25–30]. Some years later, learning this assay for  $E_2$  measurement, we used the same  $^{125}I$ - $E_2$  standard and  $E_2$  antibody developing a highly sensitive assay for plasma  $E_1S$  measurement. The procedure involved taking the samples through multiple purification steps (Fig. 1), hydrolysis and finally conversion of unconjugated  $E_1$  into  $E_2$  [31].

Taking this approach further, we improved our assay, allowing  $E_1$  as well as  $E_1S$  to be converted into  $E_2$ , each steroid to be measured with the same  $^{125}I$ - $E_2$  assay (Table 2). The approach involved adding minor amounts of  $^3H$   $E_2$  as well as  $^3H$   $E_1$  and  $^3H$   $E_1S$  for recovery standard including correction of the final results. As for this assay, we achieved a detection limit of 0.67 pM for  $E_2$ , 1.14 pM for  $E_1$  and 0.55 pM for  $E_1S$  [32], a significant improvement as compared to our earlier  $^3H$ -based methods [33]. Applying this assay to patients on treatment with anastrozole versus letrozole [18], we recorded a mean suppression of plasma  $E_2$  of 92.8% versus 95.2%, for  $E_1$  96.3% versus 98.8%, and for  $E_1S$  95.3% versus 98.9%, respectively [34]. It should be noted however that, even with this sensitive assay, 5 out of 12 patients had plasma levels of  $E_2$  below detection limit during anastrozole treatment; corresponding figures for letrozole was as high as 11 out of 12 [34].

Another interesting approach was taken by Dr. Klein and her team who used an ultrasensitive recombinant cell bioassay to measure estrogen levels in patients on treatment with letrozole [35]; this approach is discussed in detail in another paper in this issue.

A particular problem relates to plasma estrogen measurement for patients on steroidal aromatase inhibitors, such as exemestane.

**Table 1**

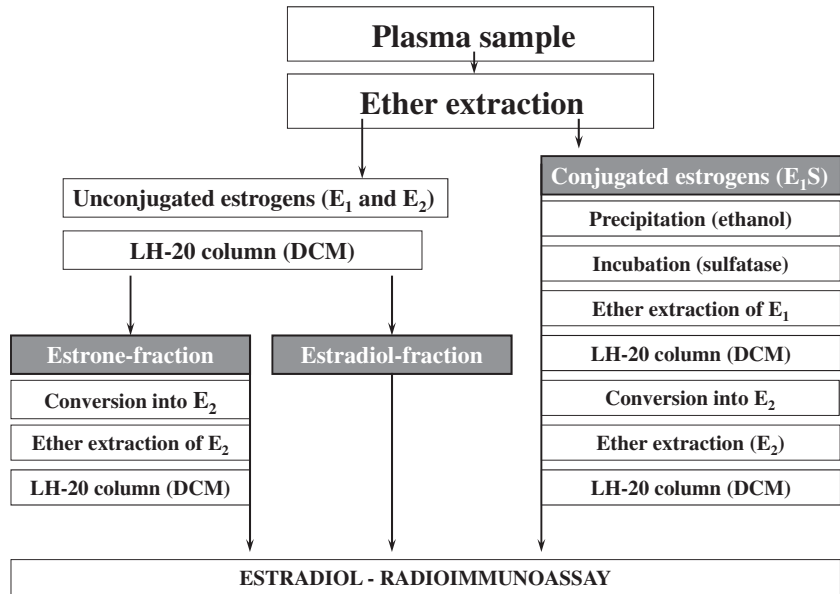
*In vivo* aromatase inhibition by different drugs evaluated in the clinical setting.

Drug	Dose	Mean inhibition	References
<i>First/second generation compounds</i>			
Aminoglutethimide (AG)	1000 mg daily	90.6%	[13]
Rogletimide	400/800/1600 mg daily	50.6%/63.5%/73.8%	[13]
Fadrozole	2 mg/4 mg daily	82.4/92.6%	[11]
Formestane*	125 mg/250 mg daily	62.3%/70.0%**/57.3%***	[14]
Formestane*	250 mg/500 mg i.m./2 w	84.8%/91.9%	[12]
Formestane*	500 mg i.m./w	91.3%	[15]
Formestane + AG	500 mg i.m./w 1000 mg daily	94.2%	[15]
<i>Third-generation compounds</i>			
Exemestane	25 mg daily	97.9%	[17]
Anastrozole	1 mg daily	96.7%	[16]
Anastrozole	10 mg daily	98.1%	[16]
Anastrozole	1 mg daily	97.3%	[18]
Letrozole	2.5 mg daily	>99.1%	[18]

\* Formestane = 4-hydroxyandrostenedione.

\*\* Administered as 125 mg b.i.d.

\*\*\* Administered as 250 mg once daily.



**Fig. 1.** Flow diagram depicting pre-purification steps required prior to determination of plasma estrogens with radioimmunoassay. Reproduced with permission from Ref. [32].

**Table 2**

Mean levels (ML), detection limits and isotope used for the radioimmuno-assays (<sup>125</sup>I or <sup>3</sup>H) with respect to different methods used at different time periods for measurement of postmenopausal estrogen levels in relation to aromatase inhibitor therapies in our laboratory.

ML	E <sub>2</sub>	E <sub>1</sub>	E <sub>1</sub> S	References
	15 pM	70 pM	400 pM	
<sup>125</sup> I:	1.3 pM 8.7%	<sup>3</sup> H: 5.3 pM 7.6%	<sup>3</sup> H: 36.1 pM 9.0%	[33]
			<sup>125</sup> I: 2.6 pM 0.7%	[31]
<sup>125</sup> I:	0.67 pM 4.4%	<sup>125</sup> I: 1.14 pM 1.6%	<sup>125</sup> I: 0.55 pM 0.14%	[32]

Mean levels (ML) = consistent with expect based on steroid disposition [54,55]. The <sup>125</sup>I radioimmunoassay was modified from original version by Dowsett and colleagues [24].

Due to potential interacting metabolites, samples collected from patients on treatment with such compounds need pre-purification with use of HPLC before radio immunoassaying [36].

Apart from differentiating between first/second generation aromatase inhibitors on the one hand as compared to the highly potent third-generation compounds on the other side, plasma estrogen measurements have been able to discriminate also between highly potent third-generation compounds like anastrozole and letrozole. Thus, two independent studies, both applying a cross-over design, have confirmed letrozole to be a more potent plasma estrogen suppressor as compared to anastrozole [30,34], consistent with tracer study findings [18]. Recently, data from the same studies have been analyzed with respect to body mass index (BMI). Conflicting data have challenged efficacy of aromatase inhibition for overweight/obese patients [37–39]; analyzing plasma estrogen levels with these sensitive radioimmunoassay's [40,41] revealed slightly higher plasma estrogen levels related to high BMI despite a similar degree of aromatase inhibition among overweight as compared to normal-weight individuals [41].

4. Tissue estrogen levels

Much interest has focused on issue estrogen levels since van Landeghem [42] and others three decades ago reported breast cancer tissue E<sub>2</sub> levels a magnitude higher as compared to plasma

levels in postmenopausal women. Thus, issues have been raised with respect to local estrogen synthesis by aromatization [43] as well as de-conjugation of E<sub>1</sub>S [44]. Using our sensitive radioimmunoassay's on tissue samples following HPLC purification (Fig. 2), we were able to detect tissue levels of E<sub>2</sub> as well as E<sub>1</sub> and E<sub>1</sub>S with high degree of sensitivity [45]. Studying tumor tissue samples collected before and during treatment with anastrozole or letrozole [34,46], we confirmed effective tissue estrogen suppression with no evidence of “escape” for any single tumor. Further, studying tissue estrogens across benign and malignant breast tissue [21], we confirmed elevated tissue to plasma E<sub>2</sub> as well as E<sub>1</sub> gradients; as for benign tissue, the tissue to plasma ratio for E<sub>2</sub> and E<sub>1</sub> averaged about 2 and 5, respectively. As for E<sub>1</sub>S, however, we found a tissue to plasma gradient averaging 0.1 only, contrasting previous findings obtained by others with use of a direct radioimmunoassay [47]. Interestingly, we confirmed elevated tumor E<sub>2</sub> levels in estrogen receptor positive but not in estrogen receptor negative tumors; these elevated levels were found positively correlated to transcriptional levels of the estrogen receptor as well as the reductive 17 hydroxy steroid dehydrogenase B7, but negatively correlated to the oxidative B2 and B12 dehydrogenases [48]. However, tissue E<sub>2</sub> and E<sub>1</sub> levels correlated even stronger to their corresponding plasma estrogen concentrations [21,48]. These findings are in agreement with the results from Professor Millers group revealing the bulk of tumor tissue estrogens to have a plasma origin [49] and the findings of Dunbier et al., reporting a strong correlation between postmenopausal plasma E<sub>2</sub> levels and tumor tissue expression of estrogen-regulated genes [50].

Based on these findings, we proposed a new hypothesis, explaining tissue to plasma hormone gradients based on physical–chemical properties for each individual compound [51]. Considering unconjugated E<sub>2</sub> and E<sub>1</sub>, these compounds are highly lipophilic, explaining a high tissue to plasma concentration gradient. In contrast, E<sub>1</sub>S is a water-soluble conjugate. While the concentration of plasma E<sub>1</sub>S exceeds the concentration of circulating E<sub>1</sub> and E<sub>2</sub> by an average factor of 8 and 40, respectively [21], this is due to the fact that most unconjugated E<sub>2</sub> and E<sub>1</sub> are converted into E<sub>1</sub>S which, on the other hand, has a plasma clearance rate of only about 10% the clearance rate of the unconjugated estrogens [52,53]. Our hypothesis does not exclude local estrogen production; nor is it inconsistent

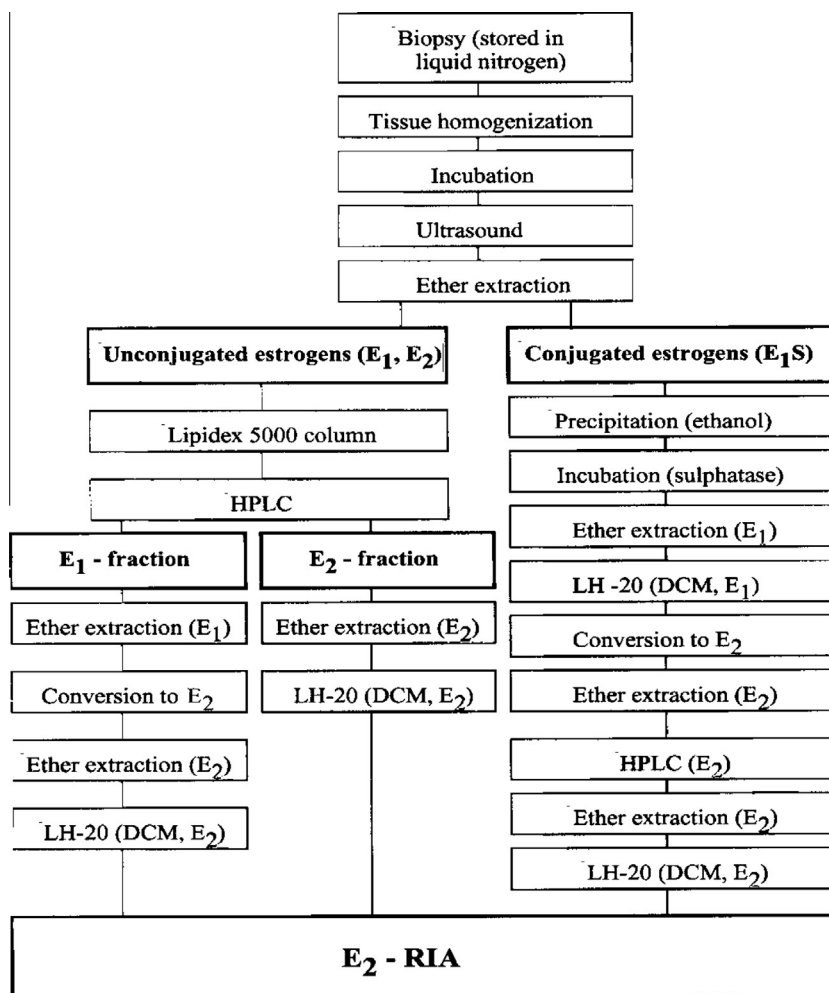


Fig. 2. Flow diagram depicting pre-purification steps required prior to determination of tissue estrogens with radioimmunoassay. Reproduced with permission from Ref. [45].

with the finding that estrogen receptor expression as well as dehydrogenase activity to some degree may influence the ratio between  $E_2$  and  $E_1$  in breast cancer tissue. However, the finding of similar tissue to plasma estrogen gradients in pre- and postmenopausal women despite substantial differences with respect to plasma estrogen levels between these groups indicate tissue to plasma equilibrium to be a rapid event [51]. Thus, local synthesis would be of minor importance to local estrogen concentration, in as much as estrogens synthesized locally would be quickly buffered by the circulating pool of hormones.

## 5. Discussion

Radioimmunoassay of plasma and tissue estrogen levels, in concert with tracer studies, has contributed significantly to our understanding of estrogen disposition. This has been mandatory to development of aromatase inhibition, currently the major endocrine treatment option for postmenopausal breast cancer patients. In contrast, there have been many publications reporting plasma estrogen levels much higher than should be assumed based on theoretical calculations [54,55] and the need for caution with respect to critically interpreting the results in the literature cannot be overemphasized [55]. Further; recent studies applying radioimmunoassays have contributed to our understanding of estrogen disposition in general, including important topics like explaining elevated tissue to plasma estrogen levels.

Measurement of plasma estrogens in postmenopausal women, due to their low levels, are time- and labor-dependent methods, involving multiple steps such as chromatographic separation and conversion [55]. The topic becomes complicated in particular when measuring estrogen levels in patients on treatment with aromatase inhibitors. For example, patients on treatment with letrozole, the most potent aromatase inhibitor currently in clinical use, will frequently have estradiol levels below the level of detectability. In contrast, as estrone-sulfate circulates at much higher levels, this steroid is usually in the detectable range in women receiving aromatase inhibitor therapy. Due to its high plasma level,  $E_1S$  has been considered a potential source for tissue estrogens through uptake and hydrolysis [56]. While  $E_1S$  may easily be hydrolyzed into unconjugated hormones, its potential contribution to tissue estrogen levels, based on arguments raised above, may be questioned. Whether plasma  $E_1S$  may be a significant contributor to tissue  $E_2$  or not, plasma  $E_1S$  may be a good proxy parameter for estrogen suppression with potent aromatase inhibitors as it exist at equilibrium with unconjugated  $E_1$  and  $E_2$  [21]. When considering tissue estrogen measurements, this becomes even more demanding, in general and requires pre-purification of samples by use of HPLC.

Taken together, while reliable radioimmunoassays are available and have played an important role in translational research, such methods are too time- and labor-demanding to be functional for routine purposes. While different liquid and gas chromatographic methods over the years gradually have improved and should be expected to replace use of radioimmunoassays for research and,



in particular, routine analysis in the future, it should be emphasized that, at this stage, there are only a few laboratories around the world with methods documented to have a detection limit allowing plasma estrogen assessment in patients on treatment with aromatase inhibitors. While such methods are expected to continuously improve, notably, they should be subject to the same strict criteria documenting sensitivity and specificity in the low concentration range with reproducibility similar to what has been shown with respect to the most sensitive radioimmunoassay's [55].

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